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## Tumor necrosis factor inhibitor: purification, NH<sub>2</sub>-terminal amino acid sequence and evidence for anti-inflammatory and immunomodulatory activities\*

The urine of some febrile patients has been shown to contain a tumor necrosis factor- $\alpha$ -inhibiting activity (TNF- $\alpha$  INH) when tested in a cytotoxicity assay using the TNF-susceptible cell line L-929. The inhibitor was purified to homogeneity using a simple three-step procedure which included a TNF- $\alpha$  affinity column, cation exchange and reverse-phase chromatography. The NH<sub>2</sub>-terminal amino acid sequence of the inhibitor showed no sequence similarity with proteins in the data bases used. Using gel filtration, it was shown that TNF- $\alpha$  and the inhibitor form a stable complex which eluted with a molecular weight of about 75 000. This value corresponds to the sum of the inhibitor (~30 000) and TNF- $\alpha$  (~45 000–50 000) molecular weight. The TNF- $\alpha$  INH blocked prostaglandin E<sub>2</sub> production by dermal fibroblasts in a dose-dependent manner, providing evidence for antiinflammatory activity. TNF- $\alpha$  INH also blocked class I antigen expression in a dose-dependent manner as measured using the human Colo 205 tumor cell line. Furthermore, TNF- $\alpha$  INH affected TNF- $\alpha$  synergism with IFN- $\gamma$ -induced HLA-DR antigen expression but had no effect on IFN- $\gamma$  activity. The data presented demonstrate that TNF- $\alpha$  bioactivity can be regulated at the protein level.

### 1 Introduction

TNF- $\alpha$ , originally described as a monocyte product with anti-tumoral activity both *in vivo* and *in vitro*, is now reported to have multiple biological activities [1–3]. Thus, TNF- $\alpha$  plays a key role in inflammation, by stimulating PGE<sub>2</sub> and collagenase production [4], and in tissue remodelling [5, 6]. In addition, TNF- $\alpha$  causes cachexia and anemia [7] and enhances HLA-A,B,C and HLA-DR gene expression in various human tumor cell lines [8]. The restriction elements function as recognition structures for antigen-specific CTL and for the initiation of the immune response [9, 10].

TNF- $\alpha$  exerts its cellular responses by binding to high-affinity cell surface receptors which have been recently characterized [11, 12]. Although TNF- $\alpha$  induces multiple biological effects, it can also trigger target cells to synthesize cytokines which, in turn, mediate additional biological effects. For example, TNF- $\alpha$  has been shown to induce the release of IL 1, granulocyte-macrophage CSF, IL 6 and platelet-derived growth factor. TNF- $\alpha$  also serves as an autocrine immunomodulator, activating macrophages and

enhancing their cytotoxic potential *in vitro* [13–16]. In turn, other cytokines such as IL 1 can induce TNF- $\alpha$  [17].

The understanding of cytokine regulation has been further complicated by the discovery of inhibitors of IL 1 and TNF- $\alpha$  [18, 19]. We previously reported that the urine of some febrile patients contained a TNF- $\alpha$  inhibiting activity (TNF- $\alpha$  INH) when tested in a cytotoxicity assay on the TNF-susceptible cell line L-929 [19]. The partially purified TNF- $\alpha$  INH shows specificity for TNF- $\alpha$  as it does not inhibit IL 1  $\alpha$  or IL 1 $\beta$  activity. It does, however, inhibit TNF- $\beta$  activity to a small extent [20]. TNF- $\alpha$  INH appears to act by directly binding to TNF- $\alpha$  [20] and may therefore be identical to the material under investigation by others [21, 22]. We have used this property to purify the TNF- $\alpha$  INH to homogeneity. The NH<sub>2</sub>-terminal sequence of the purified protein was determined. Evidence for the direct interaction of TNF- $\alpha$ /TNF- $\alpha$  INH was obtained from gel filtration studies.

In this report we also show that TNF- $\alpha$  INH regulates TNF- $\alpha$  inflammatory and immunomodulatory properties by affecting PGE<sub>2</sub> production by dermal fibroblasts and HLA class I and IFN- $\gamma$ -induced HLA class II antigen expression in the Colo 205 human cell line. The aforementioned TNF- $\alpha$  bioactivities are, thus, shown to be modulated at the protein level by the TNF- $\alpha$  INH.

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Abbreviations: TNF- $\alpha$  INH: TNF- $\alpha$ -inhibiting activity TFA: Trifluoroacetic acid

### 2 Material and methods

#### 2.1 Reagents and media

Trifluoroacetic acid (TFA), HPLC grade was obtained from Pierce (Kontron, Basel). Water and acetonitrile HPLC grade were from J. F. Baker Chemicals BV (Deventer, The Netherlands). FCS, penicillin, streptomycin, L-glutamine, MEM, DMEM and RPMI 1640 were obtained from Gibco

(Paisley, Scotland). Recombinant human TNF- $\alpha$  (hrTNF- $\alpha$ ) with a sp. act. of  $9.6 \times 10^8$  U/mg [23] and hrTNF- $\gamma$  with a sp. act. of  $3 \times 10^7$  U/mg were produced in *E. coli* (Biogen Inc., Cambridge, MA).

Murine mAb OKIa (IgG<sub>2a</sub>) against HLA-DR was purchased from Ortho Diagnostic Systems (Raritan, NJ). Murine IgG<sub>2a</sub> for control of nonspecific staining of the cells was from Coulter Immunology (Hialeah, FL). Murine mAb to HLA-A,B,C (W6/32) was obtained from the European Collection of Animal Cell Cultures (Salisbury, GB).

## 2.2 Cell culture

The murine fibroblast cell line L-929 was maintained by passage in MEM supplemented with 100  $\mu$ g/ml penicillin, 100 U/ml streptomycin, 1% L-glutamine and 10% heat-inactivated FCS. The human foreskin fibroblasts were prepared as described previously [24, 25] and maintained in DMEM supplemented with 100  $\mu$ g/ml penicillin, 100 U/ml streptomycin, 1% L-glutamine and 10% non-heat-inactivated FCS. The cells were used for bioassay between the third and the seventh passage. The human tumor cell line Colo 205 (gift from Dr. P. Scheurich, Max-Planck Society, Göttingen, FRG) was maintained by passage in RPMI 1640 supplemented with 100  $\mu$ g/ml penicillin, 100 U/ml streptomycin, 1% L-glutamine, 10% heat-inactivated FCS and  $5 \times 10^{-5}$  M 2-ME.

## 2.3 TNF- $\alpha$ INH bioassay

Prior to bioassay, all samples were sterilized using a 0.22- $\mu$ m pore size membrane (SLG S 025BS, Millipore, Bedford, MA) and tested as previously described [19]. Briefly, L-929 cells were seeded at 25 000/well and cultured for 24 h. The cells were incubated with TNF- $\alpha$  INH, hrTNF- $\alpha$  (1.5 ng/ml) and actinomycin D (1  $\mu$ g/ml) (Sigma, St. Louis, MO). Cell lysis was determined after 18 h of incubation by staining the plates with 0.5% crystal violet. Dye uptake was estimated by using a micro-ELISA auto-reader (MR 700; Dynatech Laboratories Inc., Guernsey, Channel Islands, GB). One unit (U) of TNF- $\alpha$  INH is defined as the amount of inhibitor that blocks 50% of 200 pg/ml hrTNF- $\alpha$ -induced cytotoxicity when tested on the TNF-susceptible cell line L-929 in the presence of actinomycin D.

## 2.4 Urine collection and TNF- $\alpha$ INH purification

Urine (40 l), collected from untreated febrile patients (body temperature  $> 38.5^\circ\text{C}$ ) devoid of urinary infections, was concentrated by an Amicon ultrafiltration apparatus, H10 PS-20 cartridge, molecular size cut-off of approx. 5000 (Amicon Corp., Lexington, MA). The concentrate (600 ml) was precipitated with 80% saturation of ammonium sulfate and the pellet dissolved in 150 ml of 10 mM Tris-HCl, pH 7.4, containing 2 mM EDTA.

A TNF- $\alpha$  affinity column was prepared as described previously [20]. Briefly, 1 mg hrTNF- $\alpha$  was coupled to 2 ml of Mini Leak Agarose (Kem En Tec Biotechnology Corp., Hellerup, Denmark) in 0.8 M phosphate buffer, pH 8.6,

and remaining active groups blocked by incubation in 0.1 M ethanolamine-HCl, pH 8.5. The column was equilibrated with 10 mM Tris-HCl, pH 7.4, containing 2 mM EDTA. Concentrated urine was applied to the affinity column. Bound protein was eluted with 0.2 M glycine-HCl, pH 3.5 as previously described [20]. The eluted fractions (1 ml) were immediately adjusted to pH 7.0 by the addition of 5 to 45  $\mu$ l of 1 M Tris base. The fractions containing TNF- $\alpha$  INH were pooled and concentrated by lyophilization. The lyophilized protein was dissolved in 2 ml of 50 mM sodium acetate buffer, pH 5.0, dialysed against the same buffer and applied to a Mono-S column (0.5  $\times$  10 cm) (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. Bound proteins were eluted with a linear gradient of 0–0.75 M NaCl in column buffer at a flow rate of 1 ml/min. Fractions (1 ml) were collected and tested for TNF- $\alpha$  INH. The fractions containing TNF- $\alpha$  INH activity were lyophilized. The lyophilized protein was reconstituted with 2 ml 0.1% TFA (Fluka, Buchs, Switzerland) and applied to a Pro PRC reverse-phase FPLC column (0.5  $\times$  20 cm) (Pharmacia) equilibrated in 0.1% TFA. Bound proteins were eluted with a 0%–100% acetonitrile gradient in 0.1% TFA at a flow rate of 0.3 ml/min. To each fraction (0.75 ml) 10  $\mu$ l of 0.5 M  $\text{NH}_4\text{HCO}_3$  was added. Neutralized protein was lyophilized and then dissolved in 1 ml of 10 mM Tris-HCl, pH 7.4, containing 2 mM EDTA. This material was used for bioassay and is referred to as purified TNF- $\alpha$  INH.

Fractions which revealed a single band of 33 kDa by SDS-PAGE were fractionated by microbore RP-HPLC using a Hewlett Packard model 1090 liquid chromatograph (Palo Alto, CA) fitted with a model 1040A diode array detector. Fractions were collected manually into 1.5 ml polypropylene tubes and stored at  $-20^\circ\text{C}$ . HPLC separations were done using a Brownlee Labs RB3000 cartridge column (Aquapore RP300, octyl, 7  $\mu$ m particle size, 30 nm pore size, 2.1  $\times$  220 mm; ABI, Foster City, CA) equilibrated in 90% solvent A and 10% solvent B. A gradient of solvent B from 10% to 30% and from 30% to 70% was applied sequentially for 20 and 30 min respectively, at 0.2 ml/min. Solvent A was 0.1% (v/v) aqueous TFA and solvent B was 90% (v/v) acetonitrile containing 0.1% (v/v) TFA. Fractions containing a single band with 33 kDa by SDS-PAGE were subjected to  $\text{NH}_2$ -terminal amino acid sequence analysis.

## 2.5 Amino acid sequence analysis

$\text{NH}_2$ -terminal amino acid sequence was performed using an Applied Biosystems (Warrington, GB) sequencer (ABI, Model 477A), using polybrene as a carrier. The sequence run was preceded by an external standard calibration run. Quantitation of phenylthiohydantoin (PTH) amino acids was made on the basis of peak height. PTH derivatives of amino acids were analyzed by RP-HPLC using an on-line PTH amino acid analyzer (ABI, Model 120) equipped with a PTH-C18 cartridge column (2.1  $\times$  220 mm).

## 2.6 Characterization of the TNF- $\alpha$ /TNF- $\alpha$ INH interaction

HrTNF- $\alpha$  was iodinated to a sp. act. of  $2.2 \times 10^4$  cpm/ng protein using the iodogen method [26].  $^{125}\text{I}$ -TNF- $\alpha$  (1 ng)

was chromatographed, in the presence or absence of 50 U of TNF- $\alpha$  INH by FPLC. Samples (100  $\mu$ l in PBS) were applied to a Superose 12 column (Pharmacia) equilibrated in PBS containing 1% glycerol (Fluka). The column was eluted at a flow rate of 0.2 ml/min. Fractions (0.5 ml) were collected and counted for the presence of  $^{125}$ I-TNF- $\alpha$  in a  $\gamma$ -counter (LKB, Bromma, Sweden). The column was calibrated with the following markers: dextran blue (~2000 kDa); phosphorylase b (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa);  $\alpha$ -chymotrypsinogen-A (31 kDa); soybean trypsin inhibitor (25 kDa); ribonuclease (13 kDa) and phenol red (350 dalton).

## 2.7 PGE<sub>2</sub> production by human dermal fibroblasts

Cells were seeded at 20 000/well and cultured for 48 h, then stimulated with medium alone or with hrTNF- $\alpha$  at concentrations ranging from 0.5 to 5 ng/ml. TNF- $\alpha$  INH was studied at three doses (2, 10 and 40 U/ml). After 72 h incubation, PGE<sub>2</sub> production was measured in the SN by radioimmunoassay, using an antiserum to PGE<sub>2</sub> (generously provided by Dr. L. Levine, Brandeis University, Waltham, MA) [4].

## 2.8 Immunofluorescence staining of cell surface antigens

HLA membrane expression was measured after stimulation of 5 ml of Colo 205 cells ( $2 \times 10^5$  cells/ml) cultured for 24 h prior to stimulation. Cells were stimulated with either hrIFN- $\gamma$  (10 U/ml) or hrTNF- $\alpha$  (1 to 10 ng/ml). As a control, cells were cultured in medium alone. For the indirect immunofluorescence staining of cell-surface antigens, a FITC coupled goat anti-mouse IgG<sub>1</sub> conjugate (GAM-FITC, Coulter Immunology) was used as the second antibody at a dilution of 1:40.

After stimulation, cells were washed twice in PBS and resuspended in PBS containing 5% HSA and 0.01% sodium azide. The cells ( $2 \times 10^5$ /100  $\mu$ l), were then incubated for 30 min with the indicated mAb or mouse IgG<sub>2</sub> as a control for nonspecific staining. The cells were washed twice as indicated above and incubated at 0°C with GAM-FITC for a further 30 min. Unbound GAM-FITC was removed by two washes and the cells were resuspended in 300  $\mu$ l of PBS containing 2.5% BSA. FCM analysis was performed using an EPICS 5 cell sorter (Coulter Electronics, Hialeah, FL).

The percentage of cellular HLA antigen expression was obtained by subtracting the percentage of cells stained by mouse IgG<sub>2</sub> from the initial percentage stained by either anti HLA-A,B,C or HLA-DR antibody. An addition of 17 channels of the peak of the fluorescence intensity corresponds to a twofold increase of specific immunofluorescence.

## 3 Results

### 3.1 Purification of TNF- $\alpha$ INH

TNF- $\alpha$  INH was purified from concentrated urine by a three-step procedure summarized in Table 1. TNF- $\alpha$  INH was first adsorbed on a TNF- $\alpha$  affinity column and bound material eluted with glycine buffer at pH 3.5 (Fig. 1A). This step removed the bulk of proteins which were not bound to the column and resulted in a 114-fold purification. However, fractions containing inhibitory activity were still heterogeneous when analyzed by SDS-PAGE followed by silver staining. At least three major bands were observed (data not shown). Therefore, the affinity-purified material was further purified by cation-exchange FPLC chromatography using a Mono-S column. TNF- $\alpha$  INH bound to the column and was eluted with NaCl (0.26–0.32 M), this resulted in a further 35-fold purification (Fig. 1B). As minor contaminants were still present in the inhibitory fractions, the material was lyophilized and subjected to reverse-phase FPLC chromatography. Bioactive material eluted in one major peak with 65%–70% acetonitrile (Fig. 1C). Analysis of the inhibitory fractions by SDS-PAGE under reducing and nonreducing conditions revealed a single polypeptide band migrating with an apparent  $M_r$  of 33 000. With the purification procedure described (Sect. 2.4), an overall 81170-fold purification of TNF- $\alpha$  INH was achieved with a recovery of about 3.3%. This purified protein was used for the biochemical and biological characterizations described below.

### 3.2 NH<sub>2</sub>-terminal amino acid sequence of TNF- $\alpha$ INH

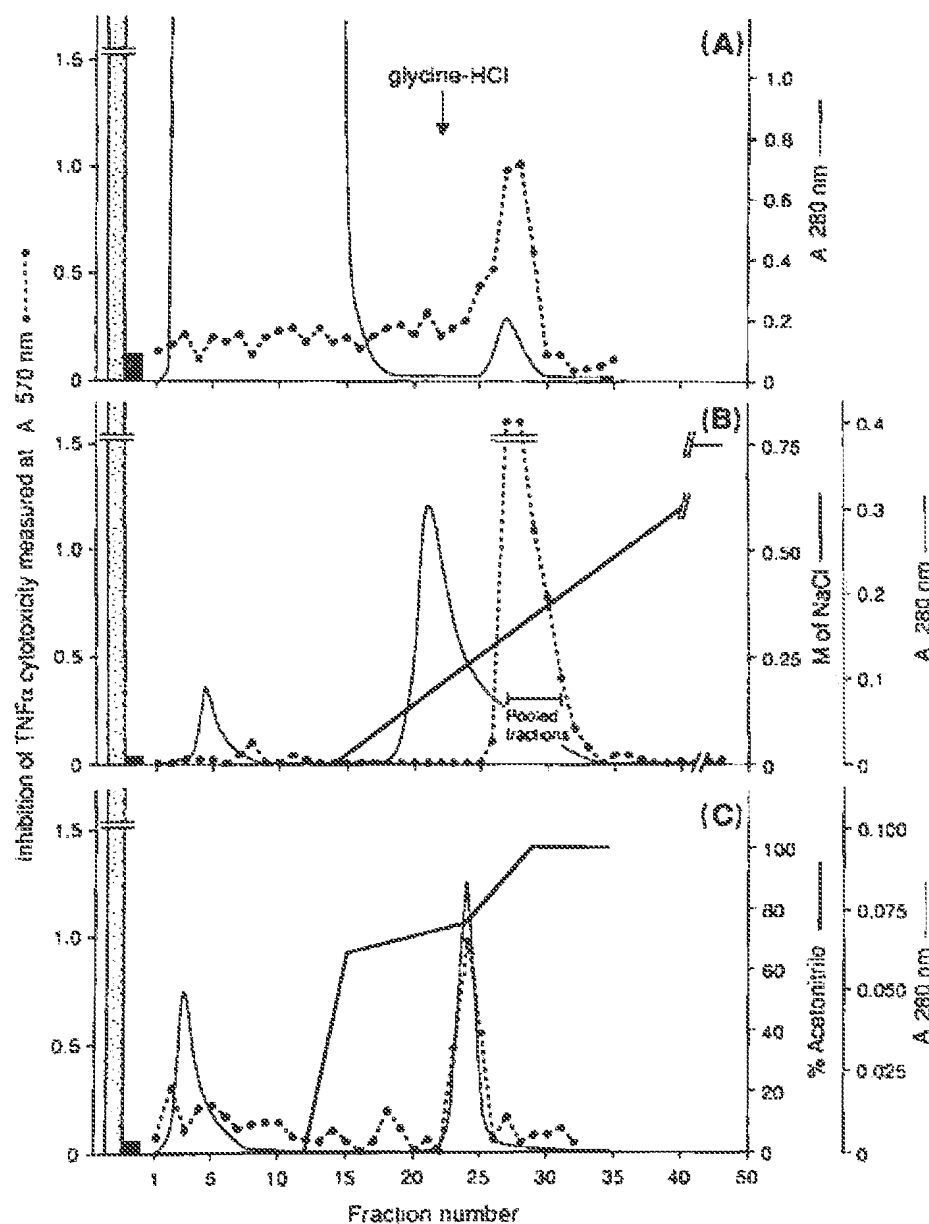
Fractions from the reverse-phase FPLC column were desalted by reverse-phase microbore HPLC. Biologically active material was located in a single peak in the HPLC profile (Fig. 2, peak B). The protein had the following NH<sub>2</sub>-terminal amino acid sequence:

Table 1. Purification of TNF- $\alpha$  INH

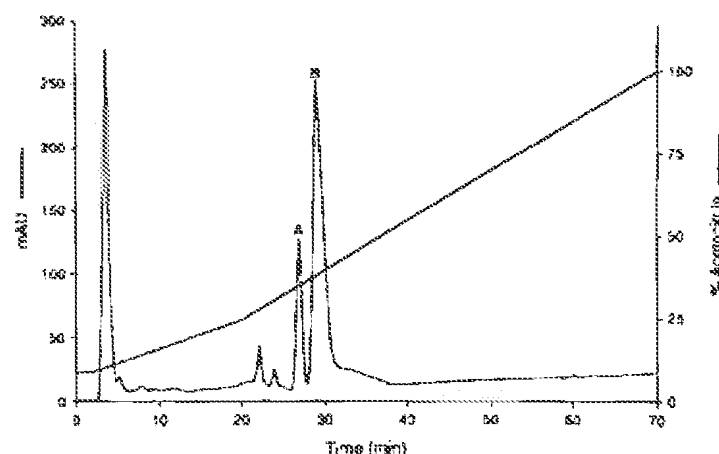
Purification step	Total protein (mg)	Volume (ml)	Activity <sup>a)</sup> (U/ml)	Recovery (%)	Purification <sup>b)</sup> factor
Urine concentrated by $(\text{NH}_4)_2\text{SO}_4$	$8.25 \times 10^3$	~550.00	140	100.0	1
TNF- $\alpha$ affinity column	0.400	33.30	720	54.0	114
Mono-S-FPLC	0.020	2.00	1350	3.5	3070
RP-FPLC	0.005	0.36	6900	3.3	81170

a) Activity was measured by a cytotoxicity assay using the TNF- $\alpha$ -susceptible cell line L-929 (see Sect. 2.3).

b) Based on the specific activity of the starting material [urine concentrated by  $(\text{NH}_4)_2\text{SO}_4$ ].



**Figure 1.** Purification of the TNF- $\alpha$  INH. (A) Elution profile of TNF- $\alpha$  affinity column. TNF- $\alpha$  INH adsorbed on the affinity column was eluted as described in Sect. 2.4. Column fractions were sterilized and tested at 1:50 dilution against hrTNF- $\alpha$  (1.0 ng/ml) in the presence of actinomycin D (1  $\mu$ g/ml) using a cytotoxicity assay. Bars represent, as also in (B) and (C), cell lysis measured in the presence of actinomycin D ( $\square$ ) and actinomycin D plus hrTNF- $\alpha$  ( $\blacksquare$ ), respectively. Values represent cell lysis measured by dye uptake at 570 nm ( $\diamond$ ). Protein elution is recorded at A<sub>280</sub> nm. (B) Elution profile of Mono-S FPLC cation-exchange chromatography. Inhibitory fractions from the affinity step were loaded onto a Mono-S FPLC cation-exchange column and run as described in Sect. 2.4. Column fractions were tested at 1:25 dilution against hrTNF- $\alpha$  (1.0 ng/ml) as in (A). Symbols as in (A). (C) Elution profile of reverse-phase chromatography. Inhibitory fractions from the ion-exchange chromatography step were loaded onto a Pro RPC reverse-phase FPLC column and run as described in Sect. 2.4. Column fractions were lyophilized, resuspended in 10 mM Tris-HCl, pH 7.4, containing 2 mM EDTA and tested at 1:50 against hrTNF- $\alpha$  (2 ng/ml) as in (A). Symbols as in (A).



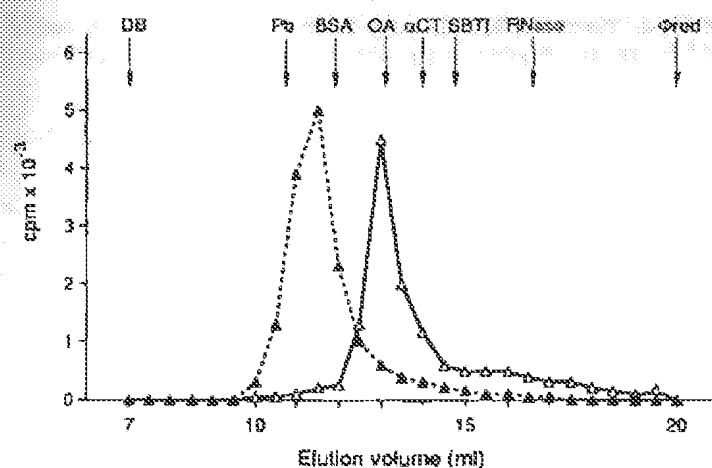
**Figure 2.** Elution profile of reverse-phase chromatography. Inhibitory fractions from Fig. 1C were loaded onto a Brownlee Labs RBS300 column and run as described in Sect. 2.4. The major peak (B) eluted at 39% of acetonitrile and was used for NH<sub>2</sub>-terminal amino acid sequence determination.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17  
D S V X P Q G K Y I H P Q X N S I

Residues at position 4 and 14 were not identified but probably correspond to cysteine residues. Analysis of sample derivatized with vinyl pyridine would be required to confirm this.

### 3.3 TNF- $\alpha$ INH/TNF- $\alpha$ interaction

Purification of the TNF- $\alpha$  INH was carried out using a TNF- $\alpha$  affinity chromatography. It is reasonable, therefore, to assume molecular recognition between TNF- $\alpha$  and the inhibitor. However, as various other proteins bound non-specifically to the affinity column, we further investigated the interaction of TNF- $\alpha$  and the inhibitor by gel filtration. TNF- $\alpha$  is a trimer of 52 kDa [27]; its molecular mass is slightly underestimated by gel filtration, values between 40–45 kDa are usually obtained. If there is a direct



**Figure 3.** Evidence of TNF- $\alpha$ /TNF- $\alpha$  INH interaction.  $^{125}$ I-TNF- $\alpha$  (1 ng) was applied in the absence or presence of 50 U of TNF- $\alpha$  INH to a Superose 12 FPLC column, calibrated and run as described in Sect. 2.6. Radioactivity of each fraction (0.5 ml) was counted in a  $\gamma$ -counter. Profiles represent cpm of  $^{125}$ I-TNF- $\alpha$  per fraction in the absence ( $\Delta$ — $\Delta$ ) or presence ( $\blacktriangle$ — $\blacktriangle$ ) of TNF- $\alpha$  INH. The elution position of the  $M_r$  markers is indicated. The abbreviations used are: DB, blue dextran; Pb, phosphorylase b; BSA: OA, OVA;  $\alpha$ CT, alpha-chymotrypsinogen; SBTI, soybean trypsin inhibitor; RNase, ribonuclease and  $\Phi$ red, phenol red.

interaction between TNF- $\alpha$  and the inhibitor,  $^{125}$ I-TNF- $\alpha$  would be expected to elute at a higher  $M_r$  value. This was demonstrated experimentally by mixing 50 U of purified TNF- $\alpha$  INH and the same amount of  $^{125}$ I-TNF- $\alpha$  and subjecting the mixture to gel filtration. Radiolabeled TNF- $\alpha$  eluted with about 75 kDa compared to a value of 45 kDa obtained when applied to the column alone (Fig. 3). The increase in mol. mass of  $\sim 30$  kDa corresponds to the mass of the TNF- $\alpha$  INH, indicating complex formation.

### 3.4 Inhibition of TNF- $\alpha$ -induced PGE $_2$ production by human dermal fibroblasts

Purified TNF- $\alpha$  INH was studied for its ability to block hrTNF- $\alpha$ -induced PGE $_2$  production by dermal fibroblasts. Human dermal fibroblasts exhibited dose-dependent PGE $_2$  production with up to 10 ng/ml of hrTNF- $\alpha$ . In contrast, when TNF- $\alpha$  INH (2, 10 and 40 U/ml) was added to fibroblasts it had no effect on the basal production of PGE $_2$  (Table 2). The PGE $_2$  production stimulated by hrTNF- $\alpha$  was inhibited by TNF- $\alpha$  INH in a dose-dependent manner. The

**Table 3.** TNF- $\alpha$  INH regulates hrTNF- $\alpha$  induction of HLA-A,B,C expression in Colo 205 tumor cell line<sup>a)</sup>

Cytokine addition		Peak of fluorescence intensity TNF- $\alpha$ INH (U/ml)		
hrIFN- $\gamma$ (U/ml)	hrTNF- $\alpha$ (ng/ml)	0	10	30
0	0	154	155	150
3	0	186	187	183
0	1	163	161	165
0	10	177	171	173
3	1	206	189	184
3	10	212	207	198

a) TNF- $\alpha$  INH or culture media alone was incubated with hrTNF- $\alpha$  (1 or 10 ng/ml) either in the absence or presence of hrIFN- $\gamma$  (3 U/ml). Values indicate peak fluorescence intensity in arbitrary units.

inhibitory activity of TNF- $\alpha$  INH appears competitive, as increasing concentrations of hrTNF- $\alpha$  reverse the inhibition. These data (summarized in Table 2) suggest that the TNF- $\alpha$  INH is capable of blocking hrTNF- $\alpha$  induced biological activity without actinomycin D and without affecting the basal level of PGE $_2$  production.

### 3.5 Inhibition of HLA-A,B,C and HLA-DR expression in human tumor cells

TNF- $\alpha$  has been reported to induce class I HLA-A,B,C molecules. In contrast to class I, class II HLA glycoproteins can only be induced by TNF- $\alpha$  in the presence of IFN- $\gamma$ . We therefore thought it important to investigate whether the TNF- $\alpha$  INH modulates TNF- $\alpha$  induced antigen class I HLA-A,B,C and class II HLA-DR expression on the human Colo 205 tumor cell line. Colo 205 cells expressed HLA-A,B,C antigen spontaneously without stimulation (Fig. 4A). After 24 h stimulation with either hrIFN- $\gamma$  (3 U/ml) or hrTNF- $\alpha$  (1–10 ng/ml) HLA-A,B,C class I expression was significantly increased. hrIFN- $\gamma$  (3 U/ml) induced a fourfold increase in class I HLA-A,B,C expression, whereas hrTNF- $\alpha$  (10 ng/ml) produced a twofold increase in antigen expression (Fig. 4A and B).

TNF- $\alpha$  INH partially blocked antigen expression when cells were stimulated by hrTNF- $\alpha$  (1 ng/ml) alone or in combination with hrIFN- $\gamma$  (3 U/ml). In contrast, antigen expression induced by a higher concentration of hrTNF- $\alpha$

**Table 2.** TNF- $\alpha$  INH blocks hrTNF- $\alpha$ -induced PGE $_2$  production by dermal fibroblasts<sup>a)</sup>

Concentration of hrTNF- $\alpha$ (ng/ml)	PGE $_2$ production by human dermal fibroblasts (ng/ml)			
	TNF- $\alpha$ INH (U/ml)			
	0	2	20	40
0	0.4 $\pm$ 0.1	1.0 $\pm$ 0.2	1.2 $\pm$ 1.1	1.5 $\pm$ 0.7
500	10.8 $\pm$ 1.9	6.4 $\pm$ 2.1	3.3 $\pm$ 0.5	1.3 $\pm$ 0.4
1000	31.8 $\pm$ 4.1	13.0 $\pm$ 3.7	2.3 $\pm$ 1.8	0.4 $\pm$ 0.2
2000	71.2 $\pm$ 12.7	11.7 $\pm$ 2.8	7.7 $\pm$ 1.4	1.7 $\pm$ 0.7
3000	76.1 $\pm$ 19.2	28.1 $\pm$ 18.3	29.1 $\pm$ 8.5	1.2 $\pm$ 0.4

a) TNF- $\alpha$  INH or buffer alone was incubated with various concentrations of hrTNF- $\alpha$ . PGE $_2$  production by cultured human dermal fibroblasts was measured after 3 days of culture. Values represent means  $\pm$  SEM ( $n = 3$ ).



(10 ng/ml) was unaffected by the addition of TNF- $\alpha$  INH (10–30 U/ml). This was not true when hrIFN- $\gamma$  was also present; partial inhibition was observed in this case. Induction of antigen expression by hrIFN- $\gamma$  was not affected by the presence of the TNF- $\alpha$  INH. Thus, when the inhibitor is added with hrIFN- $\gamma$  alone or in combination, antigen expression still occurs. The results of this section are summarized in Table 3.

Colo 205 cells express HLA-DR antigen spontaneously without stimulation (Fig. 4C). After 24-h stimulation with hrIFN- $\gamma$  (10 U/ml), HLA-DR class II expression increased about twofold. hrIFN- $\gamma$  (10 U/ml) in combination with hrTNF- $\alpha$  (10 ng/ml) induced a fourfold increase in expression (hrTNF- $\alpha$  alone had no effect). The addition of TNF- $\alpha$

Table 4. TNF- $\alpha$  INH regulates hrTNF- $\alpha$ /hrIFN- $\gamma$  synergism of HLA-DR expression in Colo 205 tumor cell line<sup>a)</sup>

Cytokine addition		Peak of fluorescence intensity TNF- $\alpha$ INH (U/ml)		
hrIFN- $\gamma$ (U/ml)	hrTNF- $\alpha$ (ng/ml)	0	10	30
0	0	109	99	99
10	0	121	122	121
0	10	101	109	107
10	10	147	131	125

a) TNF- $\alpha$  INH was incubated with combinations of hrTNF- $\alpha$  (10 ng/ml) and hrIFN- $\gamma$  (10 U/ml). Values indicate peak fluorescence intensity in arbitrary units.

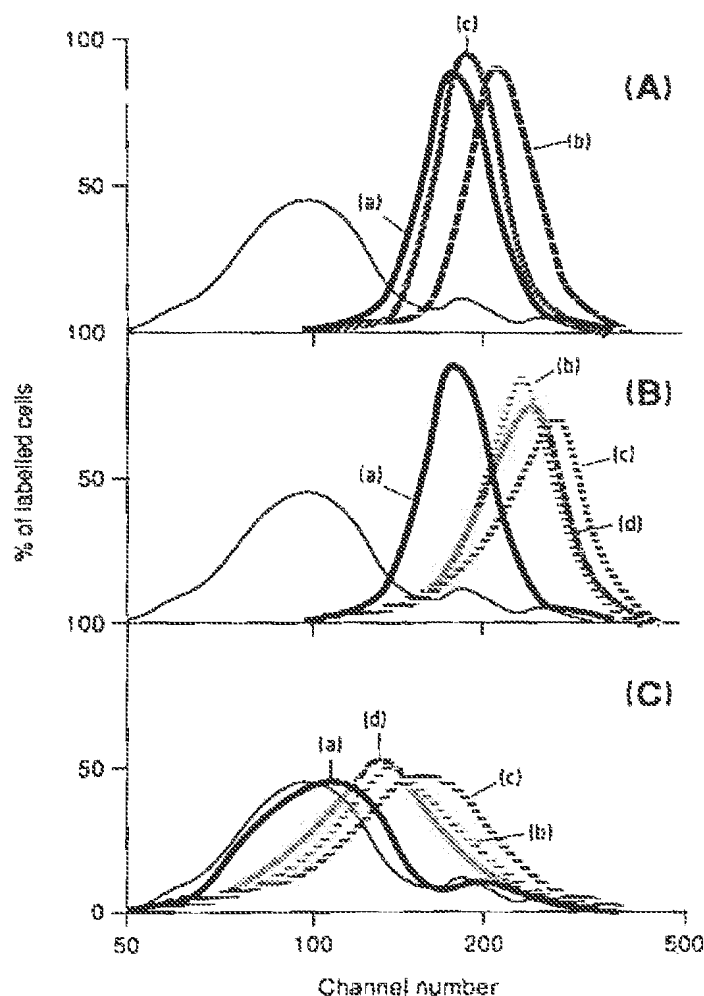


Figure 1. TNF- $\alpha$  INH regulates induction of HLA-A,B,C and HLA-DR expression in the Colo 205 tumor cell line. (A) FCM analyses of HLA class I antigen expression of Colo 205 tumor cell line in response to culture medium (a), hrTNF- $\alpha$  (1 ng/ml) (b) and hrTNF- $\alpha$  (1 ng/ml) in the presence of TNF- $\alpha$  INH (30 U/ml) (c). Nonspecific staining of the cells (—) was determined, as in (B) and (C), by using a mouse IgG<sub>2</sub>. FCM analyses of HLA class I antigen expression of Colo 205 tumor cell line in response to culture medium (a), hrIFN- $\gamma$  (3 U/ml) (b), hrIFN- $\gamma$  (3 U/ml) and hrTNF- $\alpha$  (1 ng/ml) without (c) and with TNF- $\alpha$  INH (30 U/ml) (d). (C) FCM analysis of HLA-DR class II antigen expression of Colo 205 tumor cell line in response of culture medium (a), hrIFN- $\gamma$  (10 U/ml) (b), hrIFN- $\gamma$  (10 U/ml), and hrTNF- $\alpha$  (10 ng/ml) without (c) and with TNF- $\alpha$  INH (30 U/ml) (d).

INH alone at either 10 or 30 U/ml had no effect on class II antigen expression but in cells stimulated by hrTNF- $\alpha$  in the presence of hrIFN- $\gamma$ , TNF- $\alpha$  INH inhibited expression in a dose-dependent manner (Fig. 4C). As was the case with class I antigen expression, induction of class II expression by hrIFN- $\gamma$  alone was not affected by the presence of TNF- $\alpha$  INH. Thus, when the inhibitor was added with hrTNF- $\alpha$  together with hrIFN- $\gamma$ , the antigen expression was that obtained by hrIFN- $\gamma$  alone. The results of this section are summarized in Table 4.

#### 4 Discussion

The TNF- $\alpha$  INH was purified from concentrated urine by ligand affinity chromatography followed by cation-exchange and reverse-phase FPLC chromatographies. Microbore reverse-phase HPLC was performed prior to sequence analysis to remove trace amounts of contaminating proteins. The method described herein results in about  $8 \times 10^4$ -fold of protein purification. This high purification factor may be an overestimation as the inhibitory activity in concentrated urine is partially masked by cytotoxicity [19].

The NH<sub>2</sub>-terminal amino acid sequence of the TNF- $\alpha$  INH showed no similarities with proteins in the data bases used (Swiss protein and PIR) but may be identical to the protein recently reported jointly by Olsson et al. [21] and by Engelmann et al. [22]. More extensive sequencing will be required in order to determine whether this protein is identical to the inhibitor described here.

We have previously demonstrated that the binding of <sup>125</sup>I-TNF- $\alpha$  to its receptor was inhibited in a dose-dependent manner by the addition of TNF- $\alpha$  INH [20] suggesting a direct interaction between TNF- $\alpha$  and the inhibitor. This has been confirmed by the gel filtration experiments described in this report. The dissociation constant must be quite high as a stable complex is formed that does not dissociate during gel filtration. It is most likely that the inhibitor binds to region(s) of the TNF molecule involved in receptor binding. To be effective *in vivo* at concentrations equivalent or less than TNF- $\alpha$ , it must, therefore, bind with an affinity greater or equivalent to that of TNF- $\alpha$  for its cellular receptor.

Urine from normal donors contains high concentrations of soluble, presumably processed, forms of the IL 6 and IFN- $\gamma$  receptors [28]. Hence, it is a possibility that the TNF- $\alpha$  INH is a soluble form of a TNF- $\alpha$  receptor. As the three-dimensional structure of TNF- $\alpha$  has been recently described [29] it will be most interesting to consider co-crystallization of this protein with a natural inhibitor.

We have previously shown that hrTNF- $\alpha$  induced PGE<sub>2</sub> production by dermal fibroblasts can be partially blocked by the addition of TNF- $\alpha$  INH [20]. However, as these experiments were carried out with partially purified material, we wished to eliminate the possibility that contaminants were responsible for basal PGE<sub>2</sub> production. The results summarized in Table 2 indicate that purified TNF- $\alpha$  INH does not interfere with basal level of PGE<sub>2</sub> production by dermal fibroblasts. Whether TNF- $\alpha$  inhibition of PGE<sub>2</sub>, a product with important bone resorptive activity [30, 31], is only beneficial remains to be investigated. The possibility that the TNF- $\alpha$  INH also blocks TNF- $\alpha$ -induced collagenase is at present being investigated. PGE<sub>2</sub> can exert negative feed-back regulation on tissue destruction by decreasing both IL 1 and TNF- $\alpha$  release from LPS-stimulated macrophages [32-36]. Hence, the observation that IFN- $\gamma$ , which decreases PGE<sub>2</sub> production, augments lectin-induced monocyte TNF- $\alpha$  production by five-to sevenfold [37].

It has been previously demonstrated that TNF- $\alpha$  can increase specific mRNA levels and the surface expression of HLA-A,B,C antigens in vascular endothelial cells and dermal fibroblasts [38]. TNF- $\alpha$  has also been found in some tumor cells to enhance class I, and in synergism with IFN- $\gamma$ , HLA class II antigen expression by increasing post-transcriptional gene expression [8]. HrTNF- $\alpha$  did not induce HLA-DR expression in the Colo 205 human cell line. However, hrTNF- $\alpha$  increased HLA-DR expression when the cells were pre-induced with IFN- $\gamma$ . The synergism between IFN- $\gamma$  and TNF- $\alpha$  has already been reported for antiviral [39], antiproliferative activities [40] as well as the induction of cytochrome b<sub>245</sub> heavy chain expression [41]. Addition of TNF- $\alpha$  INH to Colo 205 cells prevented class I antigen expression in a dose-dependent manner. In addition, the inhibitor blocked the synergistic effect of TNF- $\alpha$  and IFN- $\gamma$  on class II HLA-DR expression. These observations are of importance as no natural modulator of a TNF- $\alpha$  induced effect on the immune system has been reported so far.

It can be hypothesized that the absence of the TNF- $\alpha$  INH occurs in type 1 diabetes resulting in an inappropriate expression of class II antigen in the pancreatic islet  $\beta$  cells. It has been demonstrated that IFN- $\gamma$  alone is ineffective in inducing class II expression in these cells [42] and requires the presence of TNF- $\alpha$  or lymphotoxin. Whether the lack of TNF- $\alpha$  INH is responsible for the onset and the maintenance of this disease is not known.

TNF- $\alpha$ , at certain concentrations, has been shown to have anti-HIV activity [43] but, at lower concentrations, can induce expression of HIV in a chronically infected T cell clone [44]. As different levels of TNF- $\alpha$  are required to trigger one or other of these activities, one might consider whether the evolution of the disease is due to changes in

TNF- $\alpha$  INH production which in turn would regulate levels of TNF- $\alpha$ .

Our results and those of others discussed above emphasize additional control features of TNF- $\alpha$  activity in addition to those previously described, namely PGE<sub>2</sub> (32-37) and glucocorticoid [45, 46] regulation of TNF- $\alpha$  mRNA. The TNF- $\alpha$  INH, as described in this study, modulates hrTNF- $\alpha$  induced cytotoxicity, inflammatory and immunomodulating responses. The *in vivo* balance between TNF- $\alpha$  and TNF- $\alpha$  INH may be essential for homeostasis, but it will have to be established whether certain pathological situations result from a lack of TNF- $\alpha$  INH production rather than an increase in TNF- $\alpha$  production.

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